United States Environmental Protection Agency

In Vitro Aromatase Assay: Prevalidation Studies

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In Vitro Aromatase Assay:

- A cytochrome P450 enzyme complex bound in endoplasmic reticulum
- Catalyzes the conversion of androgens to estrogens
  - Androstendione ⟶ Estrone
  - Testosterone ⟶ Estradiol
- Present in ovary, placenta, testis, brain, bone, vasculature and adipose tissue
- Present in all vertebrates
- Known to be inhibited by EDCs

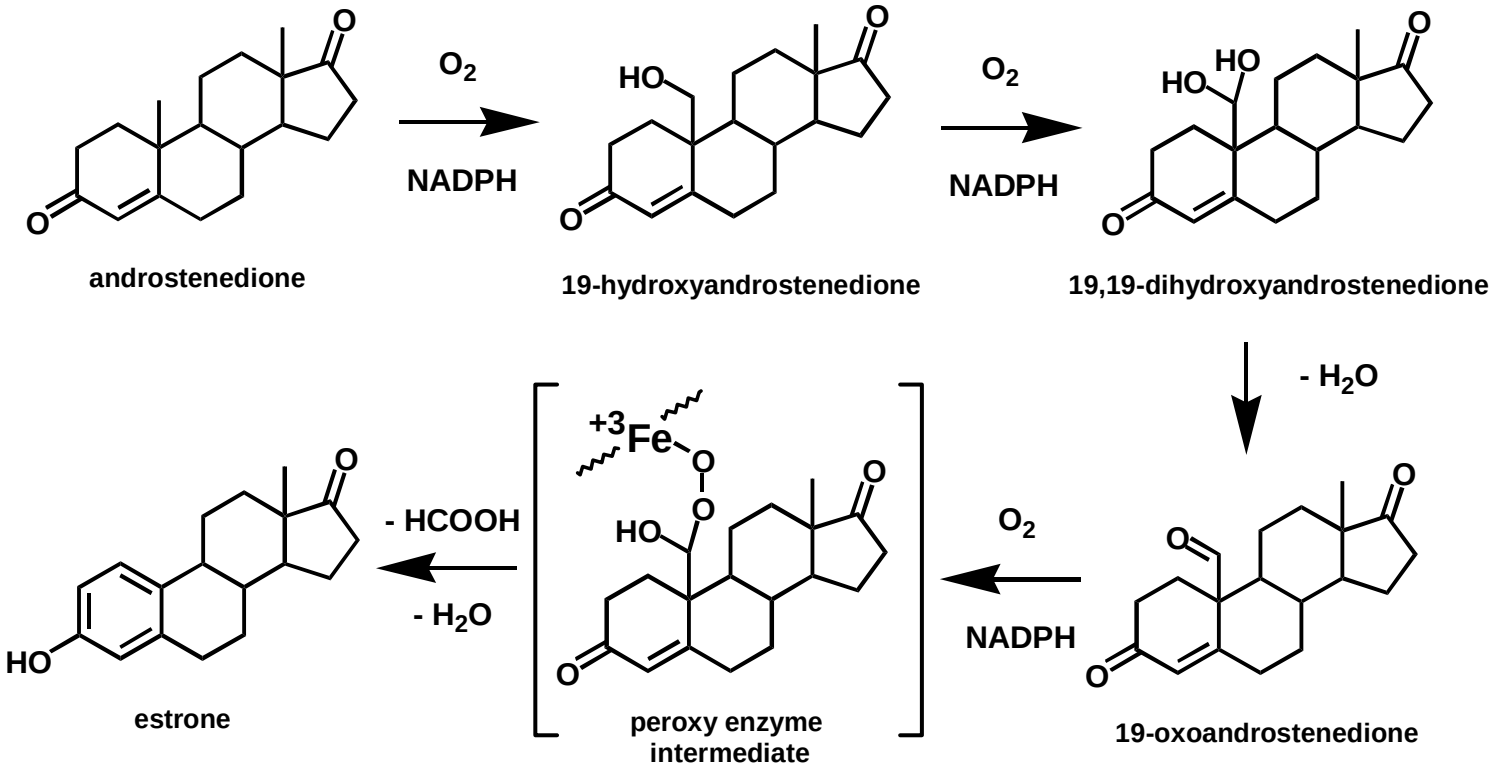
In Vitro Aromatase Assay: Prevalidation Studies

- Historical Perspective
  - EDSTAC recommended as alternative assay
  - EDSP Detailed Review Paper
    - Radiometric method
    - Human placental microsomes
  - Initial prevalidation studies
    - DRP protocol
    - Compared tissue sources for enzyme

Prevalidation Studies: Goals

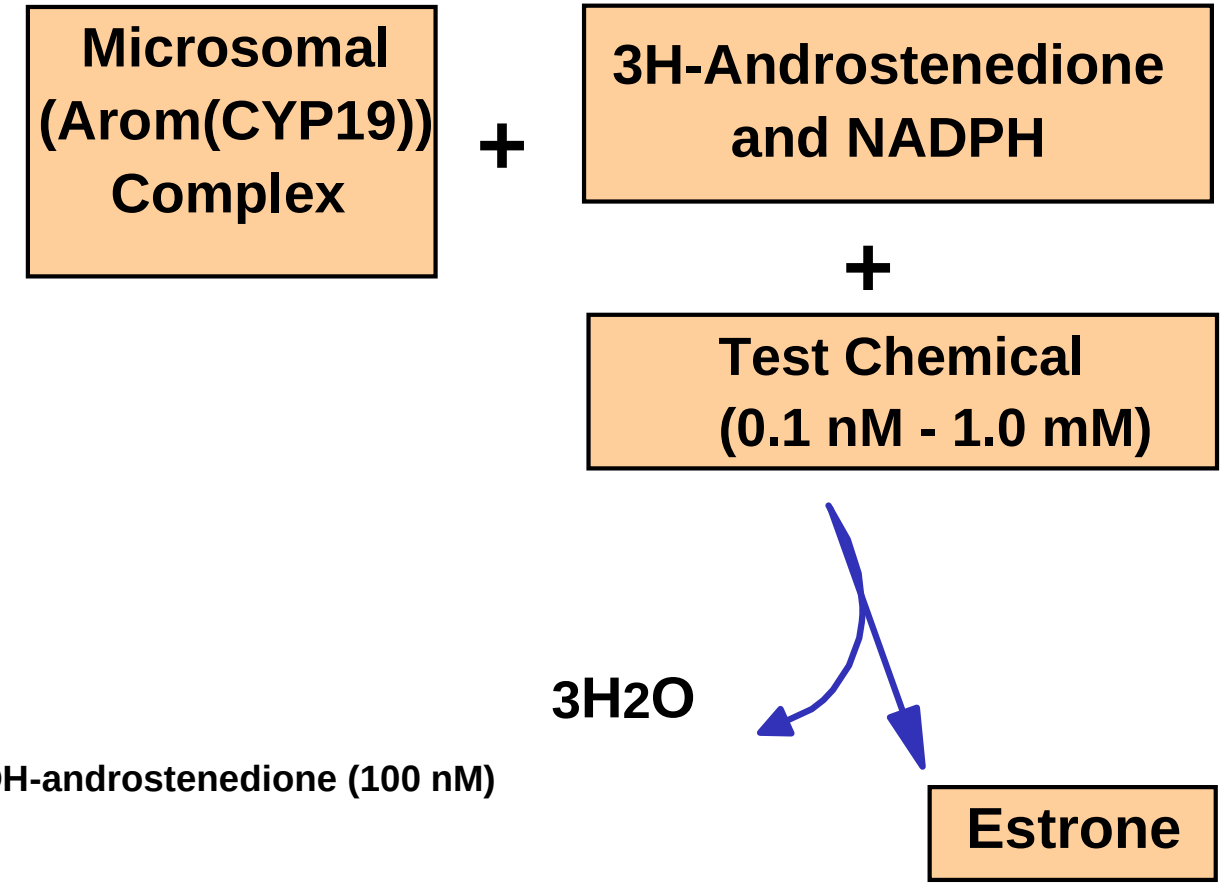
- Optimize protocols
  - Enzyme, substrate and cofactor concentrations
  - Linear time course response
  - Positive control
- Performance Criteria
  - Intra- and inter-assay variation
  - Technician variation
- Compare placental and recombinant microsomes (11 test chemicals)
- Protocol for multi-laboratory studies

Reaction Mechanism: Androstenedione to Estrone



- Cytochrome P450arom and NADPH-cytochrome P450 reductase

In Vitro Aromatase Assay: Radiometric ($^3H_20$) Method

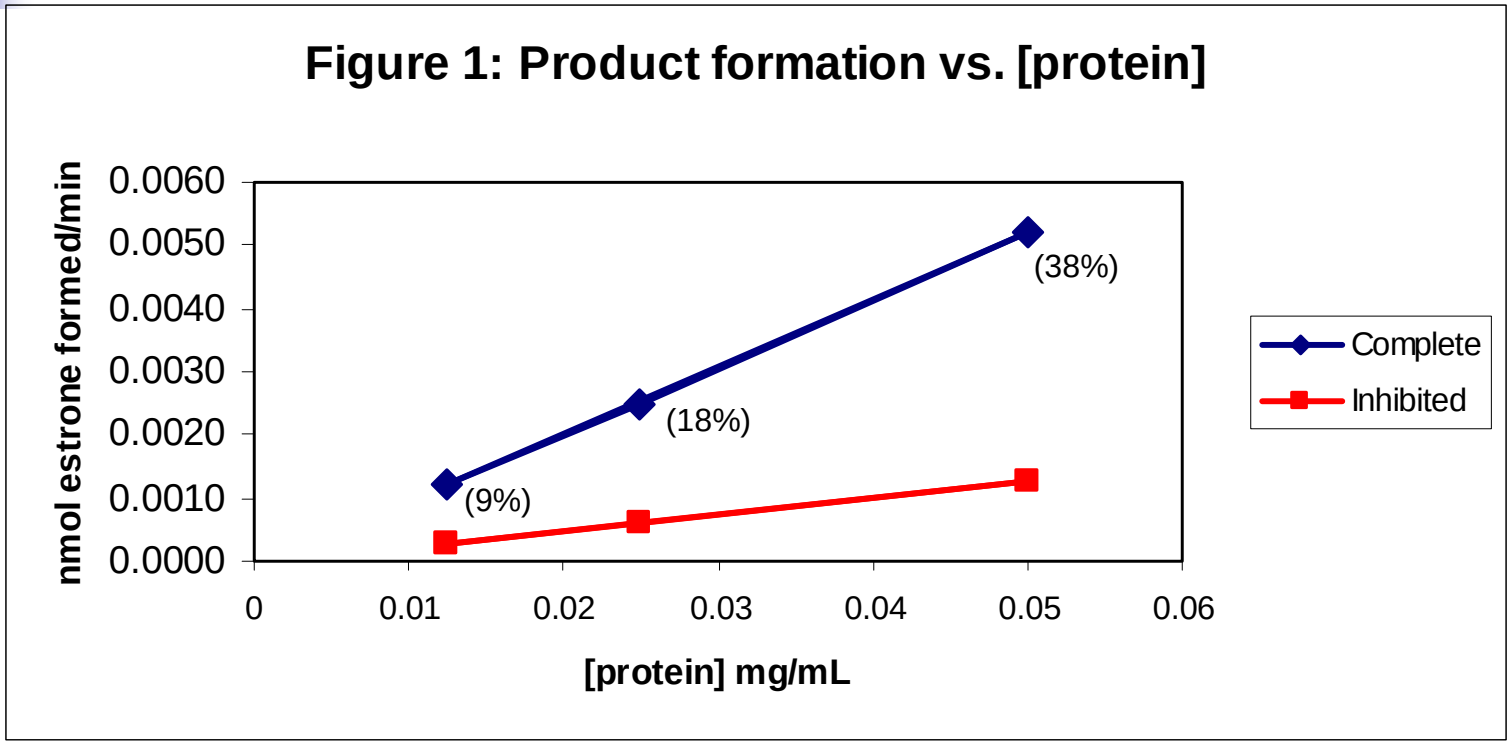


Postitive Control: 4-OH-androstenedione (100 nM)

Indicators of Optimized Protocol

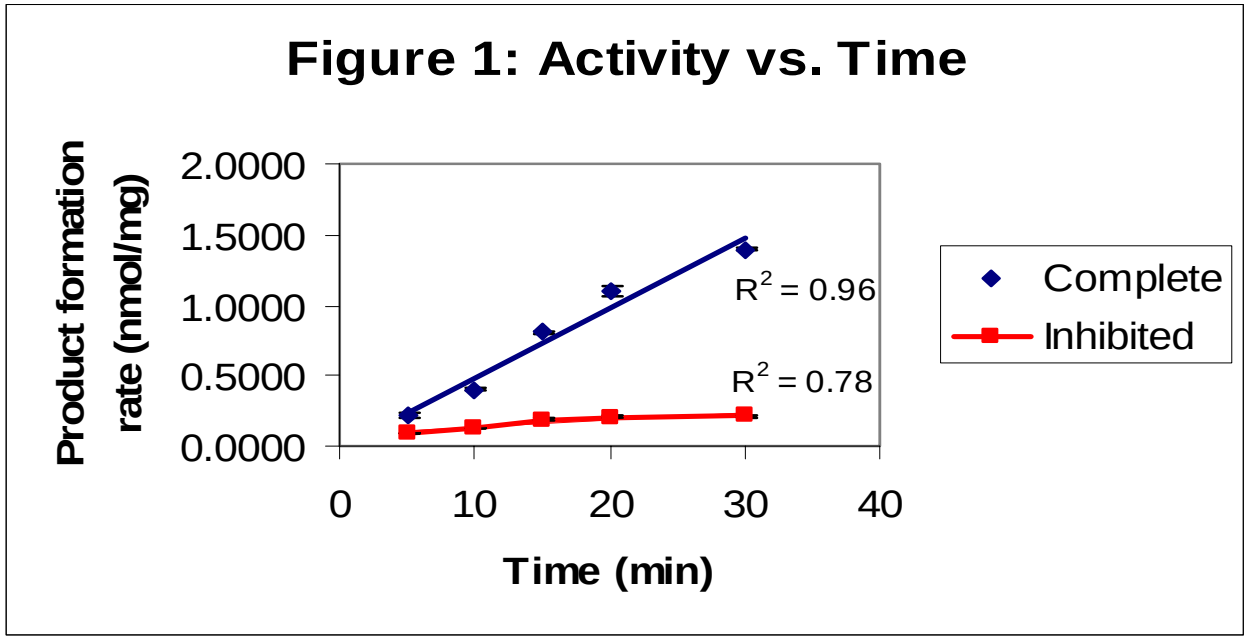
- Small fraction (10-15%) substrate converted to estrone
- Estrone production linear with time and enzyme concentration
- Estrone production dependent upon presence of enzyme and NADPH
- Estrone formation can be inhibited

Placental Microsomes: Product Formation versus Protein



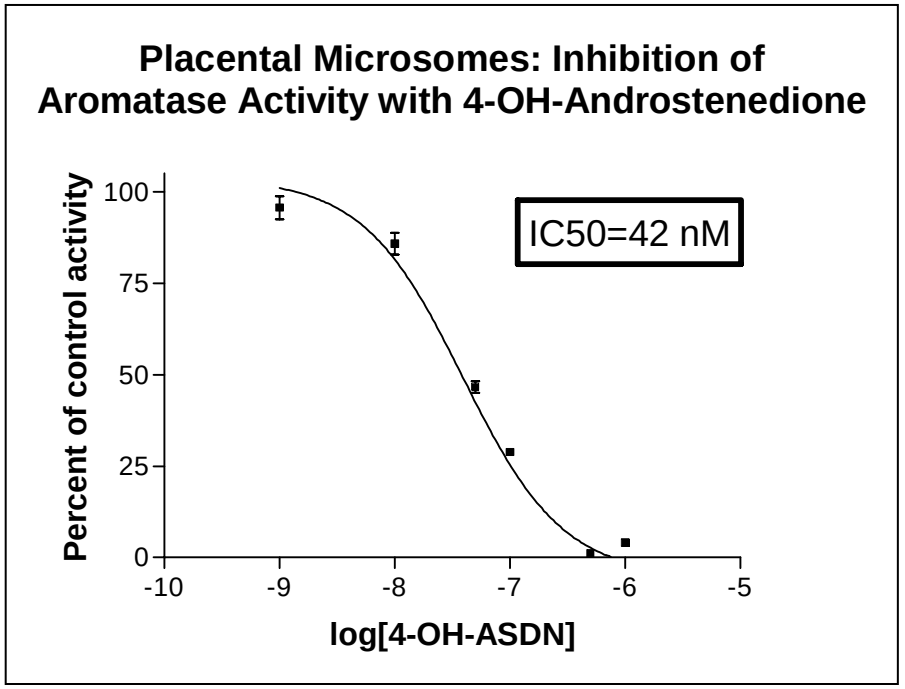
(%) conversion substrate to product
Inhibited: 4OH-androstenedione (100nM)
Intra-assay (triplicates) CV=3%; Inter-assay (3 exp.) CV=7.4%
Aromatase Optimization Supplementary Studies
(pages 11,28,29)

Placental Microsomes: Product Formation versus Time



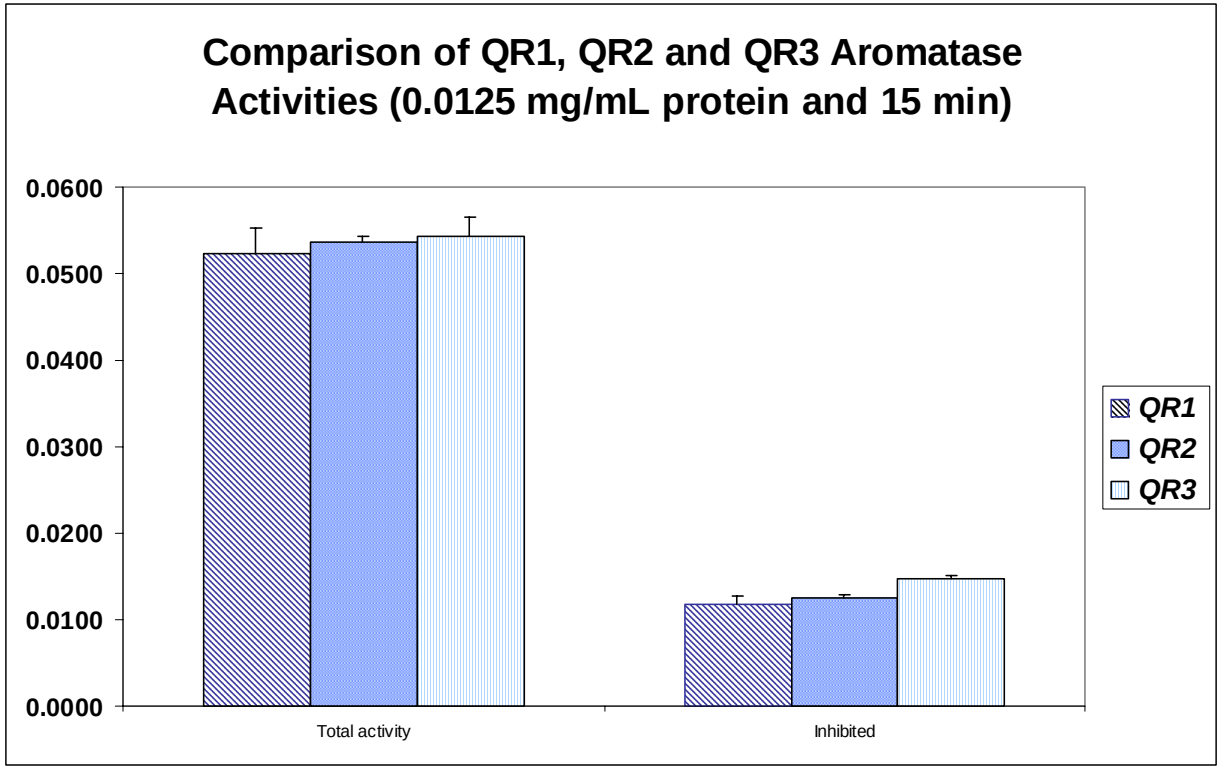
2.7, 5.1, 10, 14, 17.5% Substrate conversion over time
Inhibited= 4OH-androstenedione (100 nM)
Estrogen Production: Human Placental Assay Results
Quick Response Task 2
(Table 1, pages 2-3)

Placental Microsomes: Inhibition of Aromatase Activity



Estrogen Production: Human Placental Assay Results
Quick Response Task 3
(Figure 3)

Placental Microsomes: Intra- and Inter-Assay Variance



- Intra-assay (triplicates)

  CV 1.4-7.5%
- Inter-assay

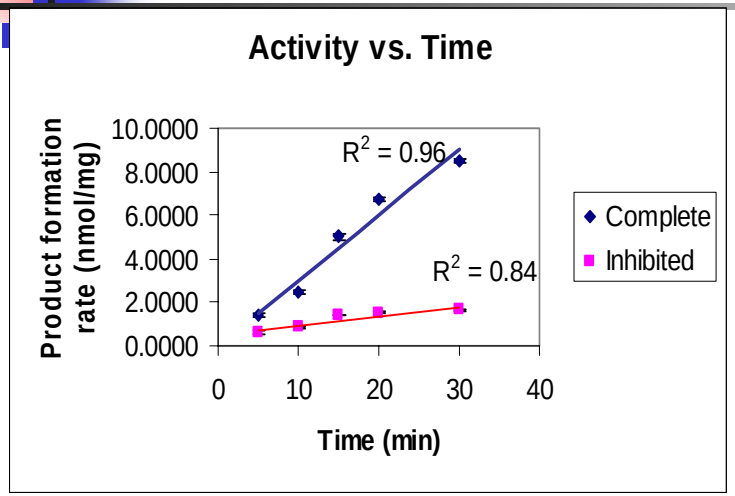
  (3 days)

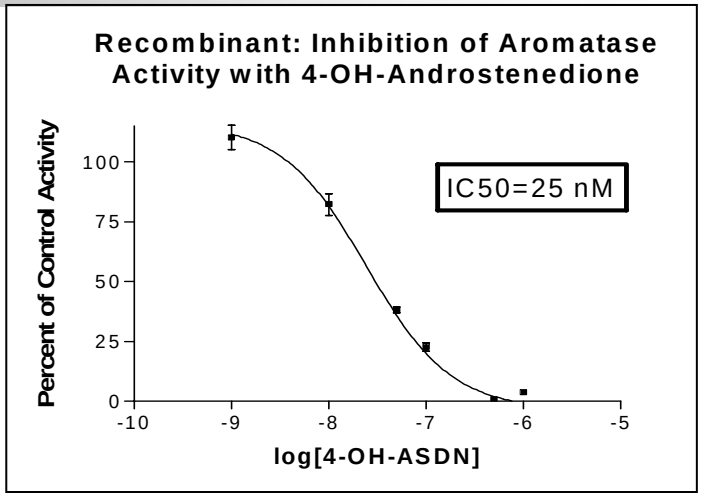
  CV 1.7 – 11.5%

Estrogen Production: Human Placental Assay Results

Quick Response Task 3 (Table 3, Figure 4)

Human Recombinant: Protocol Optimization Experiments



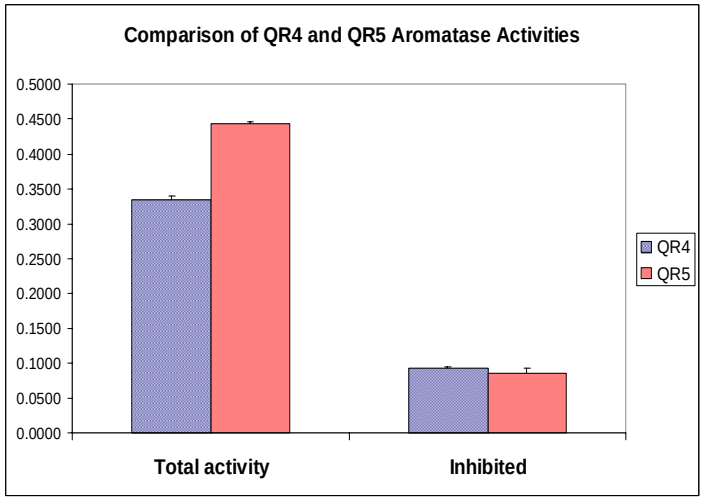


7, 12, 25, 33, 42% Substrate conversion over time

Inhibited= 4OH-androstenedione (100 nM)

Intra-assay (triplicates): CV = 1-3%

Inter-assay (2 days): CV = 5 – 20%



Estrogen Production: Human Placental Assay Results

Quick Response Task 4 and 5 (Tables 4, 5, Figures 5-7)

In Vitro Aromatase Assay: Optimized Assay Conditions

Assay Factor	Assay Type	
	Human Placenta	Human Recombinant
Protein (mg/mL)	0.0125	0.004
NADPH (mM)	0.3	0.3
[^{3}H]ASDN (nM)	100	100
Incubation time (min)	15	15
Activity (nmol/mg/min)	0.053 +/-0.001 (3)	0.283 +/- 0.0005 (2)

Optimized Protocols: Variability Between Assay Day and Technicians

- Experiment design
  - Three technicians conducted each assay independently over 3 days
  - Triplicate assay tubes
  - Maximum aromatase activity determined
  - Comparison of coefficient of variations

Estrogen Production: Human Placental Assay Results
Quick Response Task 4
Tables 7 and 8

Coefficient of Variation: Intra-assay, Assay Day, and Technician Variability

Parameter	Placenta	Recombinant
Triplicates	4%	3.7%
Tech 1	22%	17%
Tech 2	49% (12%)*	50% (11%)*
Tech 3	12%	19%
Day 3	36%	17%
Day 4	29%	30%
Day 5	47% (10%)*	53% (15%)*

(%)* CV after Tech 2, Day 5 data deleted
Estrogen Production: Human Placental Assay Results

Quick Response Task 4, Tables 7 and 8

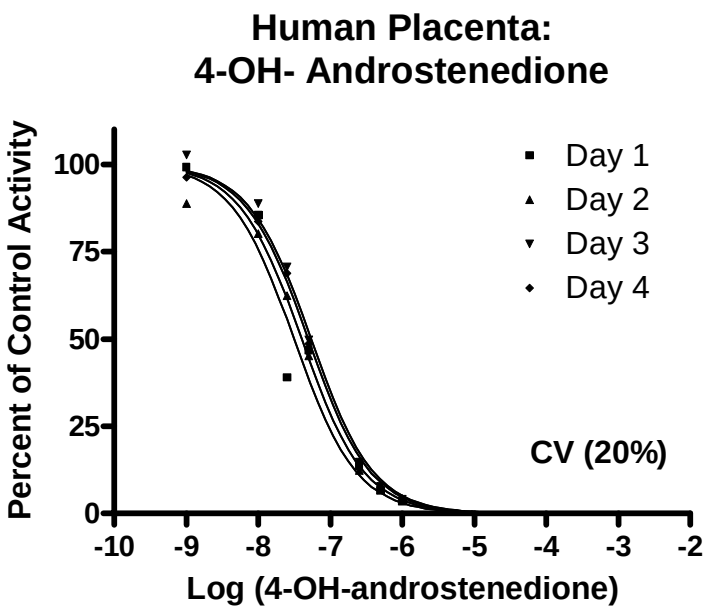
In Vitro Aromatase Assay: Comparison of Test Chemicals

- Experiment Design
  - Optimized protocols using placental and recombinant microsomes
  - Test chemicals (11, positives and negatives)
  - Complete concentration curve for each chemical ran on 4 separate days
  - Two technicians (one ran placental, the other recombinant)
  - Single set of test chemical concentrations shared by 2 tech. each day

Test Chemicals:

- Inhibitors
  - 4-OH-androstenedione
  - Chrysin
  - Ketoconazole
  - Aminoglutethimide
  - Econazole
  - Genistein (?)
- Negative for Inhibition
  - Nonylphenol
  - Atrazine
  - Bis-(2-ethylhexyl)phthlate
  - Lindane
  - Dibenz(a,h)anthracene

In Vitro Aromatase Activity: Comparison of Inhibition



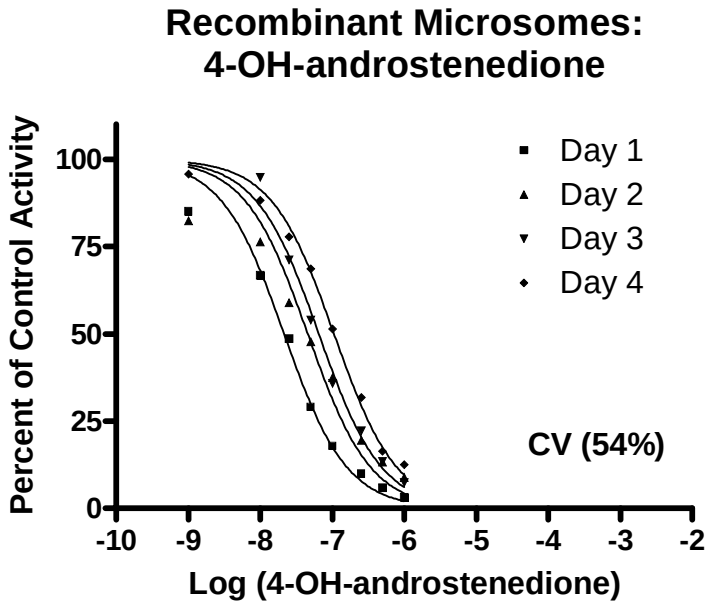
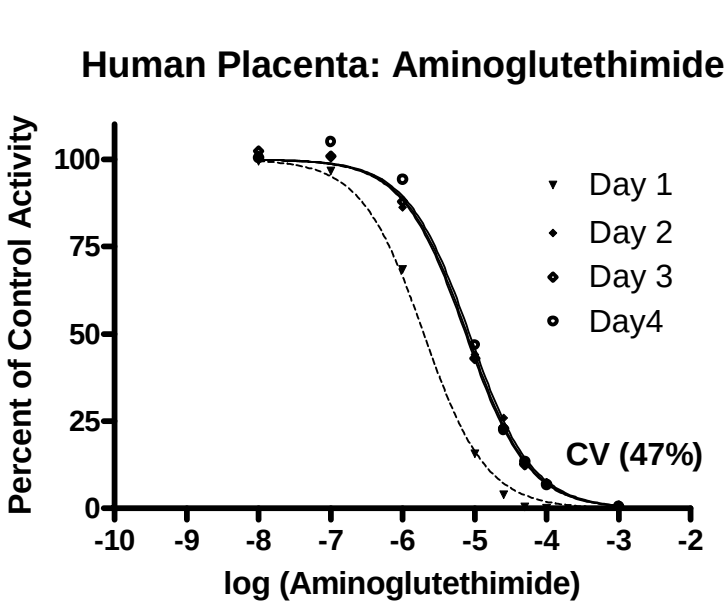


Figure 10-Placenta aromatase response curves
Figure 11-Recombinant aromatase response curves

In Vitro Aromatase Activity: Comparison of Inhibition



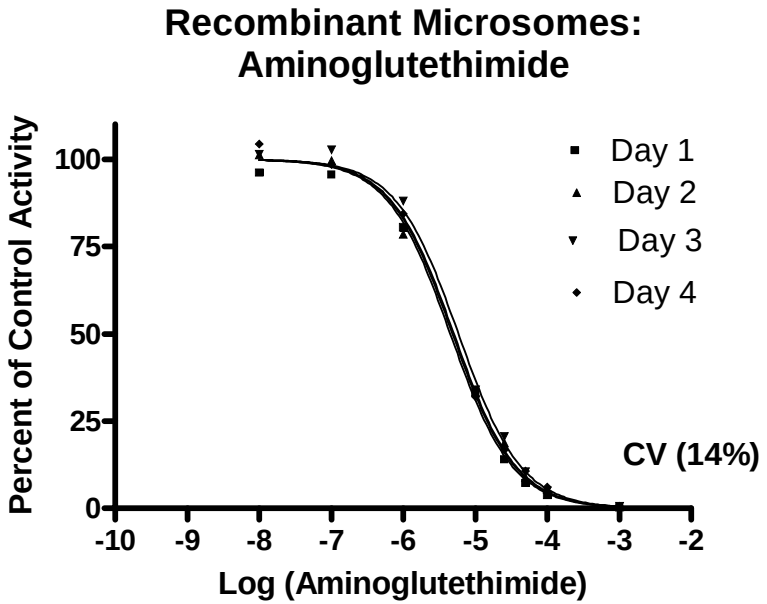
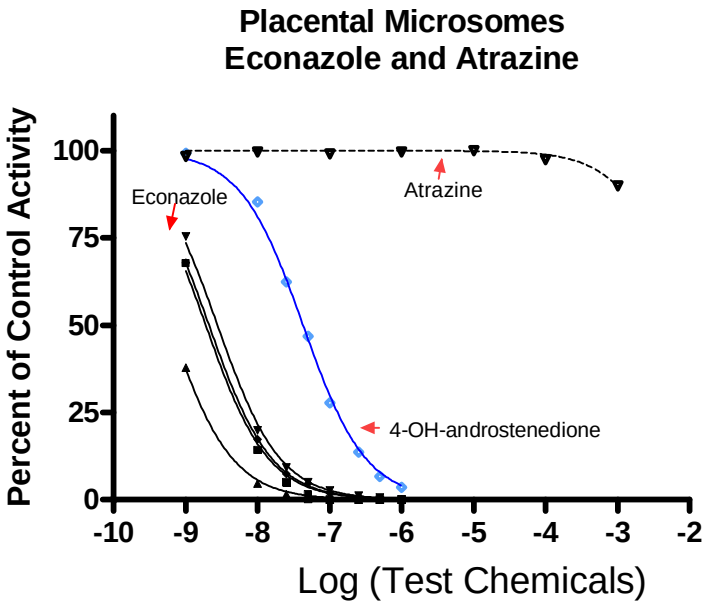


Figure 10-Placenta aromatase response curves
Figure 11-Recombinant aromatase response curves

In Vitro Aromatase Activity: Examples of Data



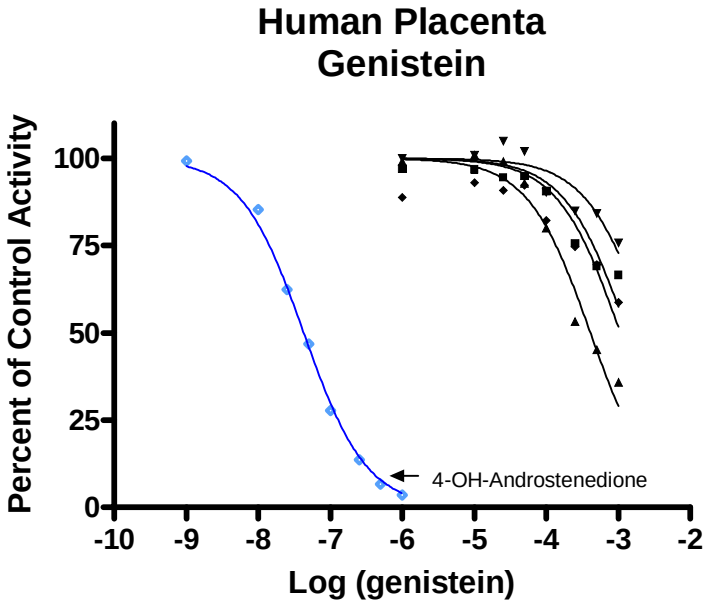


Figure 10-Placenta aromatase response curves
Figure 11-Recombinant aromatase response curves

Conclusions: Test Chemical Experiment

- Variability between reps. is greater than expected for both assays
  - IC50s for inhibitors (CVs ranged 7 – 49%)
- Technician error rather than inadequate protocol method is likely cause of variability
- Despite variability, both protocols correctly identified inhibitors

In Vitro Aromatase Assay: Next Steps

- Identify source of variability
  - Substrate concentration
  - Technician training
- Conduct additional experiment to evaluate day-to-day and technician variability (e.g, better estimate of performance criteria)
  - 2 Tech., 3 test chemicals (8-9 concentrations in triplicates), 4 days, both protocols

In Vitro Aromatase Assay: Next Steps

- Rerun assays for test chemicals with incomplete curves
  - econazole, ketoconazole
- Evaluate the usefulness of estrone measurement rather than $^{3}H_{2}0$ for recombinant protocol
- Prepare updated protocols for validation
  - Broader concentration range for test chemicals
  - Guidelines for data analysis and interpretation

In Vitro Aromatase Assay: Summary

- Protocols optimized for placenta and recombinant assays
- Assays produce similar data
- Assays differ in advantages/disadvantages
- High throughput assays
  - KGN cell line
  - CYP19/Fluorescent substrate (HTP) kit available

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